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Original Contribution

ANTIOXIDANT AND PROOXIDANT BEHAVIOR OF FLAVONOIDS: STRUCTURE-ACTIVITY RELATIONSHIPS

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Abstract—The antioxidant and prooxidant behavior of flavonoids and the related activity-structure relationships were investigated in this study using the oxygen radical absorbance capacity assay. Three different reactive species were used in the assay: 2,2′-azobis(2-amidino-propane) dihydrochloride, a peroxyl radical generator; Cu^{2+}-H_2O_2, mainly a hydroxyl radical generator; and Cu^{2+}, a transition metal. Flavonoids including flavones, isoflavones, and flavanones acted as antioxidants against peroxyl and hydroxyl radicals and served as prooxidants in the presence of Cu^{2+}. Both the antioxidant and the copper-initiated prooxidant activities of a flavonoid depend upon the number of hydroxyl substitutions in its backbone structure, which has neither antioxidant nor prooxidant action. In general, the more hydroxyl substitutions, the stronger the antioxidant and prooxidant activities. The flavonoids that contain multiple hydroxyl substitutions showed antiperoxyl radical activities several times stronger than Trolox, an α-tocopherol analogue. The single hydroxyl substitution at position 5 provides no activity, whereas the di-OH substitution at 3′ and 4′ is particularly important to the peroxyl radical absorbing activity of a flavonoid. The conjugation between rings A and B does not affect the antioxidant activity but is very important for the copper-initiated prooxidant action of a flavonoid. The *O*-methylation of the hydroxyl substitutions inactivates both the antioxidant and the prooxidant activities of the flavonoids. *Copyright © 1997 Elsevier Science Inc.*

Keywords—Flavonoid, Isoflavone, Flavanone, Flavone, Antioxidant, Prooxidant, Free radicals

INTRODUCTION

Flavonoids are diphenylpropanes (Fig. 1) that commonly occur in plants (more than 4000 flavonoids have been found) and are frequently components of the human diet. The immediate family members of flavonoids include flavones, isoflavones, and the 2,3-dihydroderivatives of flavone, namely flavanones, which are interconvertible with the isomeric chalcones. Flavanones undergo a series of transformations affecting the heterocyclic C ring to give rise to other family members of flavonoids, including anthocyanins and catechin.[1]

Some flavonoids have been found to possess antilipoperoxidant,[2] antitumoral,[3–5] antiplatelet,[6] anti-ischemic,[7] anti-allergic, and anti-inflammatory[8–10] activities. There are also reports of flavonoids inhibiting the activities of an array of enzymes, including lipoxygenase,[11,12] cyclooxygenase,[11,12] monooxygenase,[13] xanthine oxidase,[14] mitochondrial succinoxidase and NADH-oxidase,[15] phospholipase A_2,[8] and protein kinases.[16,17] These biological effects are believed to come from the antioxidant properties of the related flavonoids,[14,18–25] including their protection against iron-induced free radical reactions.[23,26,27] The inhibition of the enzymes by some flavonoids could also be due to a reaction of the flavonoid with free radicals generated at the active site of the enzymes.[28]

In contrast to the beneficial effects, some flavonoids have also been found in vitro to be mutagenic.[29–33] These harmful effects were suspected to result from the prooxidant rather than antioxidant action of the related flavonoids.[15,30–34] The biological and pharmacological effects of a flavonoid compound may depend upon its behavior as either an antioxidant or a prooxidant.

Considering the fact that many important physiolog-

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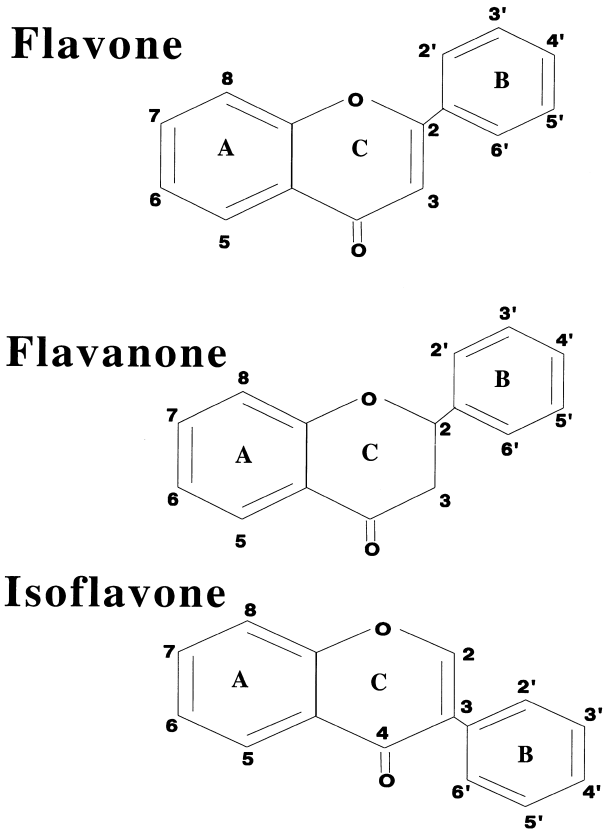

Fig. 1. Structures of the flavonoids, flavone, flavanone, and isoflavone.

ical and pharmacological functions, as well as some toxic actions, have been reported for various flavonoids, it is extremely important to understand the antioxidant and prooxidant behavior of a flavonoid and the related activity-structure relationships. The objective of this study was to elucidate the antioxidant and prooxidant behaviors of some common flavonoids and determine their activity-structure relationships as antioxidants or prooxidants, by using the oxygen radical absorbance capacity (ORAC) assay[35,36] with three different reactive species.

MATERIALS AND METHODS

Chemicals

β-Phycoerythrin (β-PE) from *Porphydium cruentum* was purchased from Sigma (St. Louis, MO, USA). The β-PE used in these experiments had the same lot number and usually lost more than 90% of its fluorescence within 30 min in the presence of 4 mmol/L of 2,2′-azobis(2-amidino-propane)dihydrochloride(AAPH). We have found that different manufacturing lots behave differently in the ORAC assay, so each new lot of β-PE should be tested to determine its suitability for the assay. AAPH was purchased from Wako Chemicals USA, Inc. (Richmond, VA, USA). 6-Hydroxy-2,5,7,8-tetramethyl-2-carboxylic acid (Trolox) was obtained from Aldrich (Milwaukee, WI, USA). Flavonoids were obtained from Indofine Chemical Co., Inc. (Somerville, NJ, USA). All flavonoids were dissolved in acetone first and then diluted with buffer (1:199−399, v/v), except for hesperidin, which was dissolved in buffer directly. When acetone was used in a sample, acetone was also used in the blank and standard, although acetone itself has a very small effect on the ORAC assay.

Automated ORAC assay

The automated ORAC assay was carried out on the COBAS FARA II spectrophotometric centrifugal analyzer (Roche Diagnostic System, Inc., Branchburg, NJ, USA) with fluorescence filters.[35] Briefly, in the final assay mixture, β-PE (16.7 nM) was used as a target of free radical (or oxidant) attack, with either (a) AAPH (4 mM) as a peroxyl radical generator ($ORAC_{ROO\cdot}$ assay), (b) H_2O_2-Cu^{2+} (H_2O_2, 0.3%; Cu^{2+} [as $CuSO_4$], 9 μM) as mainly a hydroxyl radical generator ($ORAC_{OH\cdot}$ assay), or (c) Cu^{2+} (as $CuSO_4$) (18 μM) as a transition metal oxidant. Trolox (1 μM) was used as a control standard and prepared fresh daily. The analyzer was programmed to record the fluorescence of β-PE every 2 min after AAPH, H_2O_2-Cu^{2+}, or Cu^{2+} was added. The concentration of flavonoids analyzed in the assay mixture was from 0.125 to 2 μM. All fluorescence measurements were expressed relative to the initial reading. Final results were calculated using the differences of areas under the β-PE decay curves between the blank and a sample and expressed as μmol Trolox equivalents per μmol sample,[35,36] except when Cu^{2+} (without H_2O_2) was used as an oxidant in the assay. In the presence of Cu^{2+} without any H_2O_2, flavonoids acted as prooxidants rather than antioxidants in the ORAC assay (see the Results section). The copper-initiated prooxidant activity of a flavonoid sample was calculated using $[(Area_{Blank} - Area_{Sample})/Area_{Blank}] \times 100$ and expressed as prooxidant units; one unit equals the prooxidant activity that reduces the area under the β-PE decay curve by 1% in the ORAC assay.

Statistical analysis

Linear regression analyses of $ORAC_{ROO\cdot}$ activity (Y) versus flavonoid concentrations (X) were computed using MGLH in Systat for Windows (Systat, 1992). A linear fit ($Y = a_0 + a_1X$) adequately described the data as assessed by the correlation coefficient. The standard error of the y-intercept (a_0) or the standard error of

estimate of the regression coefficient (a_1) was calculated using MGLH in Systat. Data obtained for $ORAC_{OH}$· were best described by a log-normal equation of the form:

$$Y = a_0 + a_1 e^{-0.5[\ln(X/a_2)/a_3]^2}$$

where a_0, a_1, a_2, and a_3 are rate constants; ln = natural logarithm; X = flavonoid concentration (μM); and Y = $ORAC_{OH}$· activity (Trolox equivalents, μM). Data were fit to the log-normal equation using nonlinear curve fitting algorithms in SlideWrite for Windows software (Advanced Graphics Software, Inc., Carlsbad, CA, USA).

RESULTS

Peroxyl radical absorbing activity ($ORAC_{ROO}$·)

Samples of the ORAC assay kinetic data obtained from Trolox (1 μM), glutathione (GSH) (2 μM), and myricetin (0.5 μM) are presented in Fig. 2. The importance of using an area-under-curve (AUC) technique, i.e., calculating the area under the β-PE decay curve, for quantitation of activity in the ORAC assay was discussed previously by Cao and coworkers[35] and is further demonstrated in these data. With GSH there is no clear lag phase (the length of time with 100% protection of β-PE); thus, accurate computations based solely on a lag phase are difficult. Figure 2 also shows that myricetin had a much higher $ORAC_{ROO}$· activity than either Trolox or GSH.

Figure 3 presents the type of concentration-dependent data obtained from each of the flavonoids tested. Least squares regression lines were computed between flavonoid concentration and $ORAC_{ROO}$·. The best fit as assessed by the correlation coefficient was a linear line. The regression and correlation coefficients for the flavones and isoflavones that were tested are presented in Table 1. A slope (a_1 coefficient) of 1.0 in Table 1 would represent the $ORAC_{ROO}$· activity of 1 μM of the tested compound equivalent to 1 μM Trolox, a water-soluble α-tocopherol analogue. The flavones without any OH substitutions or with a single OH substitution at the 5 position had undetectable $ORAC_{ROO}$· activity. The flavones with single OH substitutions at the 3, 6, 2′, 3′, and 4′ positions had relatively low antioxidant activity, with values for the slope of less than 0.60. Flavones such as kaempherol, luteolin, quercetin, and myricetin had $ORAC_{ROO}$· activities two to four times greater than Trolox. The isoflavones—diadzein (containing two OH substitutions) and genistein (containing three OH substitutions)—also had $ORAC_{ROO}$· activities that were 1.6- and 2.4-fold greater, respectively, than Trolox (Table 1).

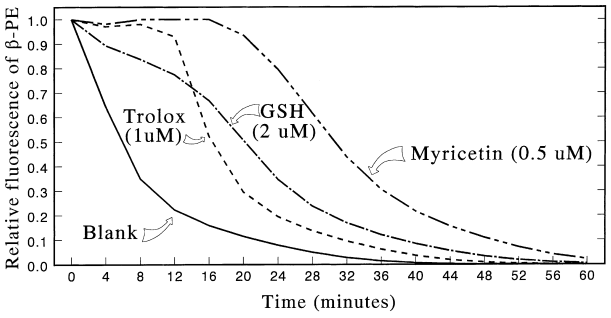

Fig. 2. Relative fluorescence of β-PE with time of incubation in the presence of Trolox (1 μM), glutathione (2 μM), or myricetin (0.5 μM).

With compounds having the same basic chemical structure, the $ORAC_{ROO}$· activity is proportional to the number of OH substitutions on the structure (Fig. 4A, B). Flavones with a single OH substitution had $ORAC_{ROO}$· activities below 1.0, whereas kaempherol, quercetin, and myricetin, which have four, five, and six OH substitutions, respectively, had $ORAC_{ROO}$· absorbing activities of 2.7, 3.3, and 4.3 (μM Trolox equivalents/μM sample), respectively (Fig. 4A). Kaempherol-

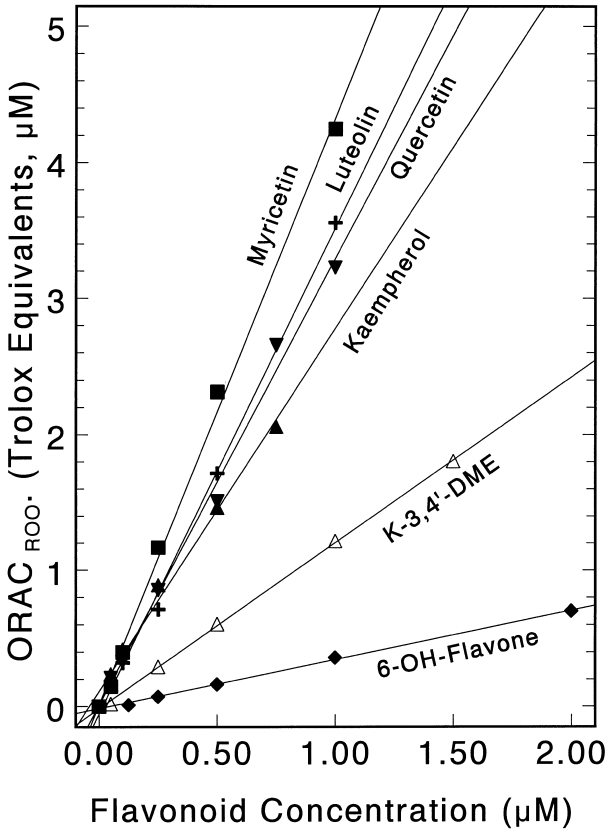

Fig. 3. $ORAC_{ROO}$· activity (Trolox equivalents, μM) as a function of concentration (μM) of 6-OH-flavone, kaempherol-3,4′-dimethylether (K-3,4′-DME), kaempherol, quercetin, luteolin, or myricetin.

Table 1. Regression Coefficients of Flavonoid Concentration (μM) (X) and $ORAC_{ROO}$. Activity (Y) (Trolox equivalents, μM)

Compound[b]	Coefficients[a]		
	a_0 (intercept)	a_1 (slope)[c]	r[d]
3-Hydroxyflavone	0.039 ± 0.030[e]	0.384 ± 0.018[f]	0.994
6-Hydroxyflavone	−0.014 ± 0.008	0.361 ± 0.008	0.999
3′-Hydroxyflavone	−0.010 ± 0.009	0.212 ± 0.007	0.999
4′-Hydroxyflavone	0.030 ± 0.025	0.603 ± 0.049	0.990
Kaempherol (3,4′,5,7-tetrahydroxyflavone)	0.110 ± 0.050[g]	2.671 ± 0.131	0.995
Kaempherol-7-neohesperidoside	0.059 ± 0.036	1.647 ± 0.039	0.999
Kaempherol-3,4′-dimethylether	−0.014 ± 0.010	1.219 ± 0.014	0.999
Luteolin (3′,4′,5,7-tetrahydroxyflavone)	−0.054 ± 0.041	3.574 ± 0.088	0.999
Quercetin (3,3′,4′,5,7-pentahydroxyflavone)	0.012 ± 0.061	3.285 ± 0.117	0.997
Myricetin (3,3′,4′,5,5′,7-hexahydroxyflavone)	0.011 ± 0.056	4.319 ± 0.119	0.998
Genistein (4′,5,7-trihydroxyisoflavone)	0.172 ± 0.095	2.375 ± 0.184	0.991
Diadzein (4′,7-dihydroxyisoflavone)	0.068 ± 0.041	1.648 ± 0.079	0.997

[a] Regression coefficients: $Y(ORAC_{ROO\cdot}) = a_0 + a_1X$(Concentration, μM).
[b] Undetectable $ORAC_{ROO\cdot}$ activity measured in the following compounds tested: flavone, 5-hydroxyflavone, kaempherol-3,7,4′-trimethylether, and luteolin tetramethylether.
[c] All a_1 coefficients significantly greater than zero ($p < .05$).
[d] Multiple correlation coefficient.
[e] Standard error of the y-intercept.
[f] Standard error of estimate.
[g] Coefficient greater than zero ($P < .10$). All other a_0 coefficients are not significantly different from zero ($p < 0.05$).

3,7,4′-trimethylether, kaempherol-3,4′-dimethylether, kaempherol-7-neohesperidoside, and kaempherol, which have one (in the 5-position), two, three, and four free OH substitutions, respectively, had $ORAC_{ROO\cdot}$ activities of 0, 1.0, 1.6, and 2.7, respectively (Fig. 4B). However, the 3′,4′ di-OH substitution is especially important to the $ORAC_{ROO\cdot}$ activity of a flavone, as demonstrated in luteolin. Luteolin, which has four OH substitutions with a 3′,4′ di-OH structure, had a much higher $ORAC_{ROO\cdot}$ activity than kaempherol, which also has four OH substitutions but no 3′,4′ di-OH structure. Luteolin tetramethylether, which has no free OH groups, showed undetectable $ORAC_{ROO\cdot}$.

The regression coefficients for the flavanone compounds are presented in Table 2. Again, undetectable

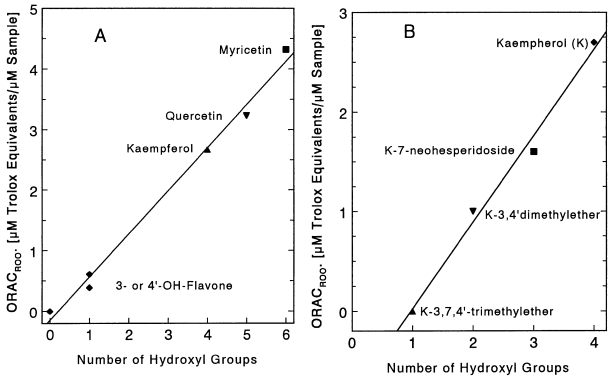

Fig. 4. Influence of the number of hydroxyl groups on the flavone molecule on measured $ORAC_{ROO\cdot}$ activity.

$ORAC_{ROO\cdot}$ activity was observed with flavanone, which has no OH substitutions on the structure. As with flavones, the flavanones with a single OH usually had a low $ORAC_{ROO\cdot}$ activity, with slopes of less than 0.3, except for 6-hydroxyflavanone, which had a value for the a_1 coefficient of 1.36. $ORAC_{ROO\cdot}$ activities of the flavanones with three to five OH substitutions were 2.6−3.9-fold greater than that of Trolox. The relationship between the number of hydroxyl groups and $ORAC_{ROO\cdot}$ activity in flavanones was curvilinear, unlike the activities with the flavone structures (Fig. 5).

Hydroxyl radical absorbing activity ($ORAC_{OH\cdot}$)

Figure 6 presents the concentration-dependent $ORAC_{OH\cdot}$ activities obtained from the flavonoids tested in this study. In all cases, the best fit was a log-normal line over the concentration range of 0−64 μM (Table 3), although at concentrations less than 5−10 μM the curve was essentially linear (Fig. 6). These data demonstrate a striking characteristic of some flavonoids in absorbing hydroxyl radicals produced in the H_2O_2-Cu^{2+} system, i.e., their $ORAC_{OH\cdot}$ activity increased proportionally to concentration at low concentrations, but after reaching a maximal value, declined with increasing concentrations. Table 3 shows the regression coefficients for the log-normal fit of flavonoid concentrations and their $ORAC_{OH\cdot}$ activities. The higher the a_2 and a_3 values in Table 3, the slower the decrease of $ORAC_{OH\cdot}$ activity with increasing concentration after the

Table 2. Regression Coefficients of Flavanone Concentration (μM) (X) and $ORAC_{ROO}$. Activity (Y) (Trolox equivalents, μM)

Compound	Coefficients[a]		
	a_0 (intercept)	a_1 (slope)[b]	r[c]
Flavanone[d]	0	0	0
6-Hydroxyflavanone	0.008 ± 0.042[e]	1.361 ± 0.044[f]	0.998
2′-Hydroxyflavanone	0.010 ± 0.022	0.297 ± 0.024	0.988
3′-Hydroxyflavanone	−0.010 ± 0.009	0.212 ± 0.007	0.995
4′-Hydroxyflavanone	0.038 ± 0.014[g]	0.249 ± 0.014	0.993
7′-Hydroxyflavanone	0.034 ± 0.015	0.129 ± 0.016	0.972
Naringenin (4′,5,7-trihydroxyflavanone)	−0.040 ± 0.024	2.669 ± 0.032	0.999
Narigin (naringen-7-neohesperidoside)	−0.055 ± 0.033	0.368 ± 0.020	0.996
Hesperidin (3′,5-dihydroxy-4′-methoxyflavanone-7-rutinoside)	0.043 ± 0.027	0.043 ± 0.005	0.976
Fustin (3,3′,4′,7-tetrahydroxyflavanone)	−0.095 ± 0.051	3.907 ± 0.099	0.999
Eriodictyol (3′,4′,5,7-tetrahydroxyflavanone)	0.187 ± 0.187	3.414 ± 0.279	0.990
Taxifolin (3,3′,4′,5,7-pentahydroxyflavanone)	0.034 ± 0.034	3.587 ± 0.065	0.999

[a] Regression coefficients: $Y(ORAC_{ROO\cdot}) = a_0 + a_1X$(Concentration, μM).
[b] All a_1 coefficients significantly greater than zero ($p < .05$).
[c] Multiple correlation coefficient.
[d] Undetectable ORAC activity measured in flavanone.
[e] Standard error of the y-intercept.
[f] Standard error of estimate.
[g] Coefficient greater than zero ($p < .05$). All other a_0 coefficients are not significantly different from zero ($p < 0.05$).

$ORAC_{OH\cdot}$ activity reached a maximal value. The maximal $ORAC_{OH\cdot}$ activities of flavones (kaempherol, luteolin, quercetin, and myricetin) and isoflavones (genistein and diadzein) were less than half of those of flavanones (fustin, taxifolin, and eriodictyol). This is shown in Figure 6 (luteolin, quercetin, and myricetin are not shown, because their activities at concentrations beyond 10 μM were not determined in this study). The flavones and isoflavones had lower values for the coefficients a_2 and a_3 than did the flavanones (Table 3), indicating a quicker decrease of their $ORAC_{OH\cdot}$ activities with increasing concentration beyond the maximal values. The $ORAC_{OH\cdot}$ activity was also related to the OH substitutions on the flavonoid structure. The flavonoids without OH substitutions (flavone and flavanone) had undetectable $ORAC_{OH\cdot}$ activity.

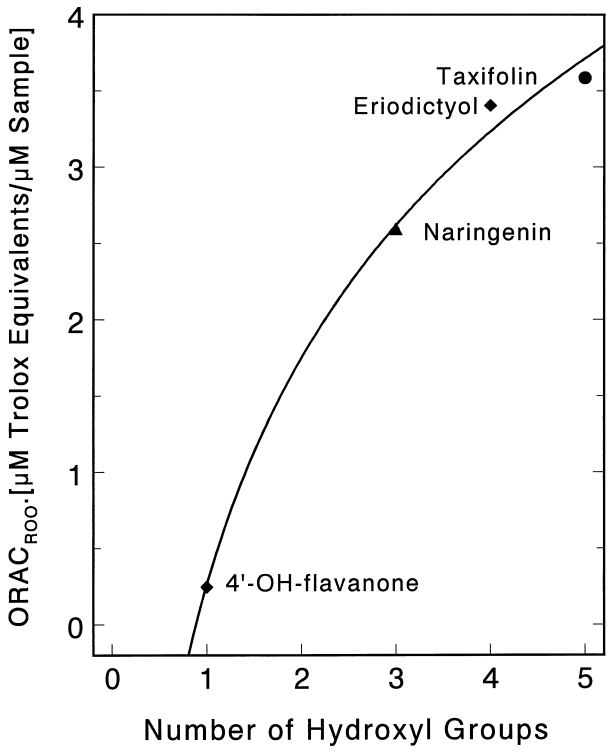

Fig. 5. Influence of the number of hydroxyl groups on the flavanone molecule on measured $ORAC_{ROO\cdot}$ activity.

The $ORAC_{OH\cdot}$ activities of these flavonoids were much weaker than their $ORAC_{ROO\cdot}$ activities, when Trolox was used as a common standard. For example, the $ORAC_{OH\cdot}$ activities of myricetin, genistein, and fustin were about 0.25, 0.25, and 0.5 μM Trolox equivalents/μM as estimated by the linear portion of the activity-concentration curve, while their $ORAC_{ROO\cdot}$ activities were 4.3, 2.4, and 3.9 μM Trolox equivalents/μM (Tables 1, 2), respectively.

Copper-initiated prooxidant activity

In the presence of Cu^{2+} without H_2O_2, flavonoids, which showed protection against peroxyl radicals and hydroxyl radicals, acted as prooxidants rather than antioxidants. Figure 7 shows the typical Cu^{2+}-initiated β-PE decay kinetics enhanced in the presence of 16 μM quercetin or myricetin. The kinetics of the prooxidant activity versus flavonoid concentration follow a rectangular hyperbola. Figure 8 and Table 4 show the linear transformation and regression between the recip-

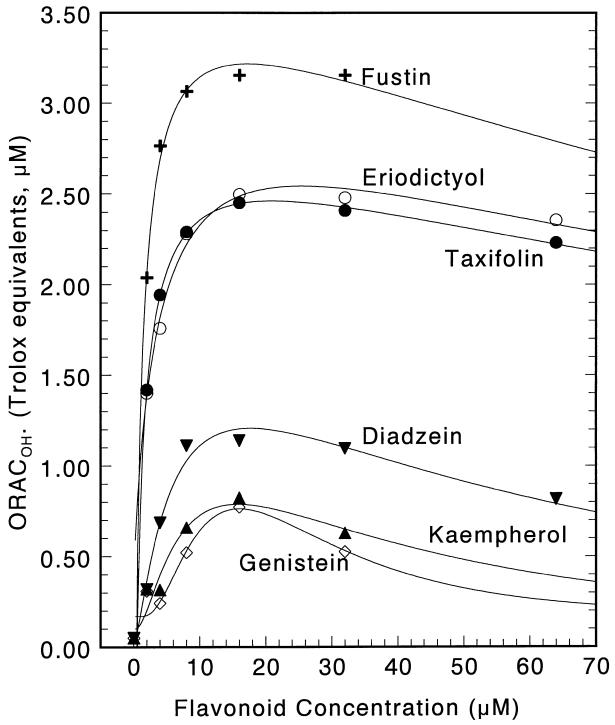

Fig. 6. ORAC$_{OH}$· activity (Y) (Trolox equivalents, μM) as a function of flavonoid concentration (μM) (X) of fustin, eriodictyol, taxifolin, diadzein, kaempherol, and genistein. Data were fit to a log-normal equation of the form: $Y = a_0 + a_1 e^{-0.5[\ln(X/a_2)/a_3]^2}$, where a_0, a_1, a_2, and a_3 are rate constants; ln = natural logarithm; X = flavonoid concentration (μM); and Y = ORAC$_{OH}$· activity (Trolox equivalents, μM).

rocal of the copper-initiated prooxidant activity (Y) and the reciprocal of concentration (X) for some common flavones and flavanones. From these equations, it is possible to calculate a maximal prooxidant activity (P_{max}) and a value equivalent to a K_m (K_m') for each flavonoid (Table 4). Here, K_m' is the concentration of a flavonoid needed for reaching 1/2 P_{max} when [Cu^{2+}] is 18 μM in the incubation medium. The copper-initiated prooxidant activity of a flavonoid depended upon the number of OH substitutions in the flavonoid structure, as with its ORAC$_{ROO}$· or ORAC$_{OH}$· antioxidant activity. With compounds having the same basic chemical structure, the copper-initiated prooxidant activity is proportional to the number of OH substitutions on the structure. Flavone and 6-hydroxyflavone, which have no or only one OH substitution, had undetectable prooxidant activity, whereas kaempherol, quercetin, and myricetin, which have four, five, and six OH substitutions, respectively, had a_1 coefficients of 0.444, 0.125, and 0.027, respectively (Table 4); the lower the a_1 coefficient (slope), the greater the prooxidant activity. Quercetin and myricetin also had maximal prooxidant activities (P_{max}) of 71 and 91 units, respectively, and K_m' of 8.9 and 2.5 μmol/L, respectively (Table 4). The P_{max} and K_m' of kaempherol were not given because the y-intercept (a_0) was not significantly different from zero. The formation of 3,4′-dimethylether or 3,4′,7-trimethylether in kaempherol (3,4′,5,7-tetrahydroxyflavone) inactivated the prooxidant activity of the flavone. Flavanone and 6-hydroxyflavanone also had no detectable prooxidant activity, while taxifolin and eriodictyol, which have four and five OH substitutions, respectively, had a maximal prooxidant activity (P_{max}) of 14 and 31 units, respectively, and a K_m' of 27.4 and 15.9 μmol/L, respectively. It is clear that the flavones (kaempherol and quercetin) had much higher copper-initiated prooxidant activities than the flavanones with the same number of OH substitutions (eriodictyol and taxifolin) (Table 4; Fig. 8).

DISCUSSION

More than two dozen different flavonoids were measured for antioxidant properties in this study. The selection of these compounds was based on chemical structure characteristics, availability, and prevalence in plant foods. All compounds were members of the family of flavonoids including flavones, isoflavones, and flavanones. Special attention was paid to the number, position, and *O*-methylation of free hydroxyl groups on these compounds. Other similar previously published studies evaluated fewer compounds in each group and did not focus on the number and modification of free hydroxyl groups.[18,19,21–24]

When free radicals and other reactive species are generated in living systems, a wide variety of antioxidants come into play. Other investigators previously reported antioxidant activities of flavonoids by using 2,2′-azinobis(3-ethylbenzothiazoline)-6-sulfonate radical cation ($ABTS^{\bullet+}$),[19] superoxide anion ($O_2^{-\bullet}$),[21] and hydroxyl radical ($OH^{\bullet}$) produced photolytically[18] or by Fe^{2+}-H_2O_2.[34] However, $ABTS^{\bullet+}$ is not a natural free radical found in the body, and in addition the related antioxidant activity assay system using $ABTS^{\bullet+}$ has some deficiencies.[35] The antioxidant activity of some flavonoids against $O_2^{-\bullet}$, which is not a strong oxidant, has been analyzed by using the inhibition percentage of the $O_2^{-\bullet}$ signal in ESR spectra after 30 s incubation of the flavonoids with H_2O_2, KOH, and acetone.[21] This is only a semiquantitative assay system. The $OH^{\bullet}$ is the most reactive free radical found in the body, but it has a very short half-life. Results obtained with some common flavonoids by using $OH^{\bullet}$ produced photolytically[18] are also not consistent with results obtained by using $OH^{\bullet}$ produced by Fe^{2+}-H_2O_2.[34]

The ORAC assay developed recently by Cao and coworkers[35,36] provides a unique and novel way of eval-

Table 3. Regression Coefficients for Log-Normal Fit of Flavonoid Concentration (μM) (X) and $ORAC_{OH}$. Activity (Y) (Trolox equivalents, μM)

	Coefficients[a]				
Compound	a_0 (intercept)[b]	a_1[c]	a_2[c]	a_3[c]	r^2
Flavone					
Kaempherol	0.1 ± 0.1*	0.7 ± 0.1*	15.8 ± 3.4*	1.1 ± 0.3*	0.94
Isoflavones					
Diadzein	0.04 ± 0.07	1.2 ± 0.1*	17.6 ± 1.3*	1.4 ± 0.1*	0.99
Genistein	0.2 ± 0.1	0.6 ± 0.1	16.0 ± 2.7*	0.7 ± 0.2	0.90
Flavanones					
Taxifolin	−37 ± 188	40 ± 188	20.9 ± 0.4*	10.2 ± 24.4	0.99
Fustin	−130 ± 15,702	133 ± 15,702	17.2 ± 4.0*	16.4 ± 971.6	0.99
Eriodictyol	0.4 ± 1.0	2.1 ± 1.0	25.4 ± 2.6*	2.0 ± 0.7	0.99

Log-normal equation: $Y = a_0 + a_1 e^{-0.5[\ln(X/a_2)/a_3]^2}$, where a_0, a_1, a_2, and a_3 are rate constants; ln = natural logarithm; X = flavonoid concentration (μM); and $Y = ORAC_{OH}$. activity (Trolox equivalents, μM).

[a] Coefficients significantly different from zero ($p < 0.05$) are indicated by *.

[b] Intercept ± standard error of the y-intercept.

[c] Coefficient ± standard error of estimate.

uating the potential antioxidant activity of various compounds and biological samples. This method is superior to other similar methods for two reasons. First, the ORAC assay system uses an area-under-curve technique and thus combines both inhibition time and inhibition degree of free radical action by an antioxidant into a single quantity.[35] Other similar methods[37–40] use either the inhibition time at a fixed inhibition degree or the inhibition degree at a fixed time as the basis for quantitating the results. Second, different free radical generators or oxidants can be used in the ORAC assay. This is important because the measured antioxidant activity of a compound depends upon which free radical or oxidant is used in the assay.[41]

In the current study, we successfully used the ORAC assay to determine the antioxidant and prooxidant behavior of flavonoids and related structure-activity relationships with three different oxidants, including AAPH (an $ROO^{\bullet}$ generator), Cu^{2+}-H_2O_2 (mainly an $OH^{\bullet}$ generator), and Cu^{2+} alone. $ROO^{\bullet}$ is a common free radical found in the body that is slightly less reactive than $OH^{\bullet}$ and thus possesses an ''extended'' half-life of seconds instead of nanoseconds.[42] The ORAC assay using AAPH measures all traditional antioxidants including ascorbic acid, α-tocopherol, β-carotene, glutathione, bilirubin, uric acid, and melatonin,[36,43] while the ORAC assay using Cu^{2+}-H_2O_2

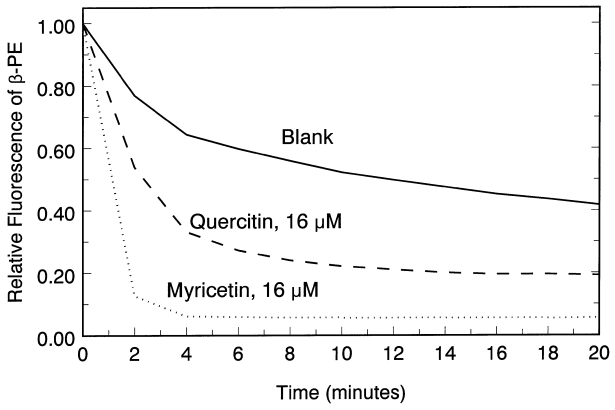

Fig. 7. Relative fluorescence of β-PE with time of incubation in the presence of 18 μM Cu^{2+} and selected flavonoids.

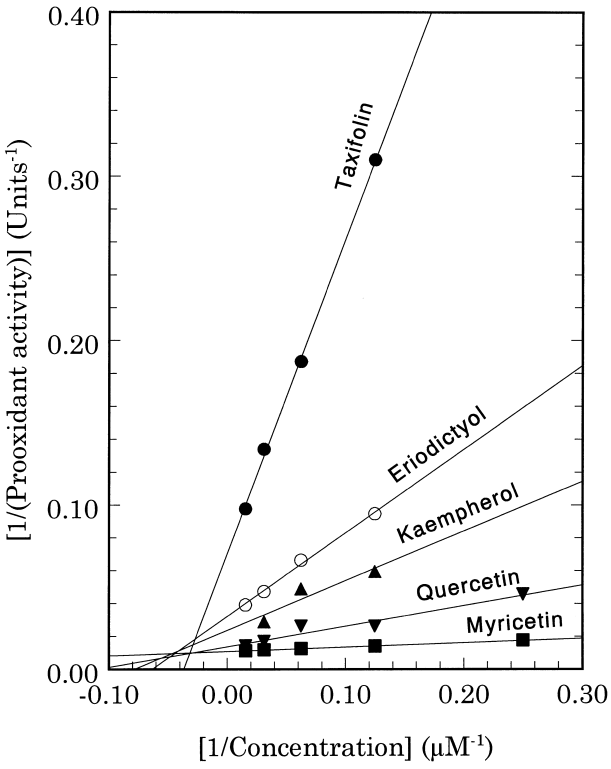

Fig. 8. Linear regression of (1/Prooxidant activity) (Y) and (1/Concentration [μM]) for selected flavones and flavanones.

Table 4. Regression Equations of Prooxidant Activity^{-1} (Y) and Flavonoid Concentration^{-1} (X) When Cu^{2+} Was Used as a Reactive Species Generator in the ORAC Assay

Compound	Coefficients[a]		r^2	P_{max}[b]	K'_m[c]
	a_0 (intercept)	a_1 (slope)			
Flavone					
Kaempherol	0.002 ± 0.005[d,e]	0.444 ± 0.037[f]	0.980	—[g]	—[g]
Quercetin	0.014 ± 0.002	0.125 ± 0.018	0.943	71	8.9
Myricetin	0.011 ± 0.000	0.027 ± 0.001	0.998	91	2.5
Flavanones					
Taxifolin	0.070 ± 0.003	1.916 ± 0.045	0.999	14	27.4
Eriodictyol	0.032 ± 0.002	0.509 ± 0.024	0.996	31	15.9

Regression equation: $Y = a_0 + a_1X$.
[a] A smaller value for the a_1 coefficient indicates greater prooxidant activity.
[b] P_{max}: maximal prooxidant activity (units).
[c] K'_m: the concentration of a flavonoid needed for reaching 1/2 P_{max}.
[d] Coefficient not significantly different from zero ($p > 0.05$).
[e] Standard error of the y-intercept.
[f] Standard error of estimate.
[g] The values are not determined because a_0 is not significantly different from zero.

measures compounds like mannitol, glucose, uric acid, and transition metal chelators, but not ascorbic acid and α-tocopherol.[44–46] When AAPH is used as a peroxyl radical generator in the ORAC assay described in this study, the analyzed flavonoids are not likely to affect the rate of radical generation from AAPH because (a) chemically, it is unlikely that the -OH on a flavonoid reacts with -N=N- in AAPH (R-N=N-R) and inhibits the formation of N_2 and the release of R radicals at 37°C, and (b) even if the -OH on a flavonoid could react with AAPH, the inhibition of the release of radicals from AAPH by the flavonoid is not possible, because the molar ratio of AAPH to the flavonoid in the ORAC assay system was very high (2000–32,000). However, when other investigators[23,26,27,34] have used a transition metal and/or H_2O_2 as an oxidative stressor in evaluating the antioxidant activity of flavonoids, the flavonoid may react directly with the transition metal and affect the rate of related free radical generation. This is why we performed the ORAC assays using both Cu^{2+}-H_2O_2 and Cu^{2+} alone in this study. H_2O_2 and transition metals such as Cu^{2+} are potentially available in vivo and are frequently used in vitro to induce oxidative damage to protein and nucleic acids.[47,48]

The possibility that flavonoids would affect the sensitivity of β-PE to the free radical damage from AAPH can also be excluded in this study, although some flavonoids inhibit the activities of an array of enzymes,[8,11–17] suggesting an interaction between β-PE and these specific enzyme proteins. Flavonoids, at different concentrations (0.125–2 μM in this study), afforded complete protection of β-PE against AAPH for certain periods of time, after which the kinetics of the damage to β-PE were basically the same as in the blank (see the lag phase produced by myricetin in Fig. 2). The complete protection was also linearly correlated with the concentration of all tested flavonoids (Tables 1, 2). If the complete protection was due to a flavonoid-induced loss of sensitivity of β-PE to peroxyl radical damage, the loss must be a complete loss. However, it is very unlikely that, in the presence of flavonoids, the sensitivity of a protein (β-PE) to free radical damage was lost completely only for certain periods; furthermore, this loss would also have to be linearly correlated with the concentration of these flavonoids of different structures, and then completely recovered, if there is no direct absorption of free radicals by the added flavonoids. Second, the absorption and fluorescence properties of β-PE are exquisitely sensitive to the conformation and chemical integrity of the protein.[38] The incubation of β-PE with a flavonoid at 37°C in the absence of any reactive species does not change the fluorescence of the protein (data not shown), which is similar to observations with Trolox, ascorbic acid, and uric acid.[46] It seems likely that flavonoids would change the conformation and chemical integrity and thus the fluorescence of the β-PE protein if they decreased or increased the sensitivity of the protein to the damage caused by AAPH, Cu^{2+}-H_2O_2, or Cu^{2+} alone.

Using the ORAC assay, we clearly demonstrated that the same flavonoids could behave as both antioxidants and prooxidants, depending on concentration and free radical source. Flavonoids acted as antioxidants against free radicals but demonstrated prooxidant activity when a transition metal was available. The antioxidant activities (including $ORAC_{ROO\cdot}$ and $ORAC_{OH\cdot}$ activities) and the copper-initiated prooxidant activities of these flavonoids depended on their structures. Flavone and flavanone, which have no OH substitutions and which provide the basic chemical structures for the

flavonoids, showed neither antioxidant activities nor copper-initiated prooxidant activities. However, it is known that OH substitution is necessary for the antioxidant activity of a flavonoid.[49]

In general, the more OH substitutions, the stronger the $ORAC_{ROO\cdot}$ activity. Weak $ORAC_{ROO\cdot}$ activity (0.2–0.6 Trolox equivalents) was observed for flavones with single OH substitutions at the 3, 6, 2′, or 4′ position and in flavanones with single OH substitutions at the 7, 2′, 3′, 4′, or 7′ position. A flavone with a single OH substitution at the 5 position, however, has undetectable $ORAC_{ROO\cdot}$ activity, whereas a flavanone with a single OH substitution at the 6 position has an $ORAC_{ROO\cdot}$ activity of 1.36 Trolox equivalents, even stronger than Trolox. The former result supports reports showing that flavonoids devoid of a 5-OH substitution were more potent NADH-oxidase inhibitors,[15,50,51] provided this inhibition process is related to the antioxidant actions of flavonoids. Previous reports demonstrated that at least two OH groups must be present in the structure of flavonoids in order to protect lysosomes against oxidative stress; flavone and monohydroxyflavone were ineffective protectors.[52] With compounds having the same basic chemical structure, the $ORAC_{ROO\cdot}$ activity was proportional to the number of OH substitutions on the structure. Kaempherol, quercetin, and myricetin, which have four, five, and six OH substitutions, respectively, had $ORAC_{ROO\cdot}$ activities of 2.7, 3.3, and 4.3 Trolox equivalents, respectively (Fig. 4A). This is consistent with the inhibitory effects of these flavones on the platelet aggregation induced by ADP, collagen, and platelet activating factor: kaempherol < quercetin < myricetin.[6] The importance of 3′,4′ di-OH substitution in the B ring to the $ORAC_{ROO\cdot}$ activities of flavonoids found in this study was similar to findings of (a) antioxidant activity assays of flavonoids based on formation and decay of flavonoid aroxyl radicals[18] or on the ability of flavonoids to quench the chromogenic radical cation $ABTS^{\bullet+}$,[19] (b) protection by flavonoids of lysosomes against oxygen radicals,[52] and (c) inhibitory effects of flavonoids on the release of reactive oxygen species by stimulated human neutrophils.[53]

Unlike the $ORAC_{ROO\cdot}$ activities, which were always linearly correlated with concentration, the activities of flavonoids in absorbing hydroxyl radicals produced by Cu^{2+}-H_2O_2 increased proportionally to flavonoid concentration at low concentrations, but after reaching a maximum $ORAC_{OH\cdot}$ declined with increasing concentration. The characteristics of the $ORAC_{OH\cdot}$ activities of these flavonoids are actually due to the Cu^{2+} used in the Cu^{2+}-H_2O_2 system. In this study, we observed that flavonoids in the presence of Cu^{2+} without H_2O_2 acted as prooxidants rather than antioxidants, and this copper-initiated prooxidant activity always increased with the concentration of the flavonoids (Table 4) but in a manner that approximates a rectangular hyperbola. This finding does not support the concept of chelation by some flavonoids, including quercetin,[26,27] since addition of a chelator, like EDTA, produced very powerful antioxidant activity against Cu^{2+} in the ORAC assay using Cu^{2+} alone, but no activity was seen in the assay using AAPH, a peroxyl radical generator (data not shown).

Therefore, it is clear that flavonoids play two opposite roles when Cu^{2+}-H_2O_2 was used as a reactive species generator in the ORAC assay: (a) absorbing hydroxyl radicals and other reactive species produced by the reactions among Cu^{2+}, H_2O_2, and O_2, and (b) producing reactive species through the direct reaction among flavonoids, Cu^{2+}, and O_2. The generation of reactive species and the subsequent damage to macromolecules (target) in the flavonoids-Cu^{2+}-O_2 system can be accounted for by the following reaction sequences:

$$2Cu^{2+} + \text{F-OH} \rightarrow 2Cu^{+} + \text{F=O} + H^{+}$$

$$Cu^{2+} + O_2 \rightarrow 2CuO_2^{+}$$

$$CuO_2^{+} + \text{F-OH} \rightarrow Cu^{+} + \text{F=O} + HO_2^{-}$$

$$CuO_2^{+} + Cu^{+} + 2H^{+} \rightarrow 2Cu^{2+} + H_2O_2$$

$$\text{target-}Cu^{+}\text{complex} + H_2O_2 \rightarrow \text{target-}Cu^{2+}\text{-}HO^{\bullet} + OH^{-}$$

$$\text{target-}Cu^{2+}\text{-}HO^{\bullet} \rightarrow \text{damaged target} + Cu^{2+}$$

where F represents a flavonoid structure as depicted in Fig. 1.

It is the Cu^{2+}-initiated prooxidant activity of a flavonoid that makes its $ORAC_{OH\cdot}$ activity decrease with increasing flavonoid concentration after the maximal hydroxyl radical absorbing value. It is not surprising that the antioxidant activities for some flavonoids measured by using $OH^{\bullet}$ produced photolytically[18] are not consistent with results obtained using $OH^{\bullet}$ produced by Fe^{2+}-H_2O_2.[34] The much higher copper-initiated prooxidant activities demonstrated by the flavones in this study, compared with those shown by the flavanones with similar structures, indicate the particular importance of the conjugation between rings A and B of flavones in the Cu^{2+}-initiated prooxidant activities of this group of flavonoids. It also explains why, compared with the flavones, much higher maximal $ORAC_{OH\cdot}$ activities and much slower decreases of $ORAC_{OH\cdot}$ activities with concentration (after the maximal activities) were observed in the flavanones when Cu^{2+}-H_2O_2 was used as mainly an $OH^{\bullet}$ generator in the ORAC assay. Flavanones are 2,3-dihydroderivatives of flavones and

thus have no conjugation between rings A and B in their chemical structures (Fig. 1).

The copper-initiated prooxidant activity of a flavonoid, as its peroxyl or hydroxyl radical absorbing antioxidant activity, also depends on the number of free OH substitutions on its structure. The more OH substitutions, the stronger the prooxidant activity. *O*-Methylation and probably also other *O*-modifications of the flavonoid OH substitutions inactivate both the antioxidant and the prooxidant activities of the flavonoids. Ioku and coworkers[54] concluded that quercetin was a more efficient antioxidant than its monoglucosides in a test system wherein phospholipid bilayers were exposed to aqueous oxygen radicals. Luteolin was also shown by other investigators to be a significantly stronger antioxidant than its two glycosides.[55] Flavonoids occur in foods generally as *O*-glycosides with sugars bound usually at the C3 position. The inactivation of transition metal-initiated prooxidant activity of a flavonoid by methylation and glycosidic modification of the OH substitutions could have particularly important physiological and pharmacological implications. Flavonoids, such as quercetin and kaempherol, have been shown to induce nuclear DNA damage and lipid peroxidation in the presence of transition metals.[30–33] The rapid metabolic inactivation of mutagenic flavonoids catalyzed by catechol-*O*-methyltransferase has been demonstrated by Zhu et al.[56] in vivo. The action of catechol-*O*-methyltransferase could be a major reason for the lack of carcinogenic activities of some flavonoids in vivo. However, transition metal-initiated prooxidant action of a flavonoid may also be responsible for changes in regulation of enzyme activities by the flavonoid.

The flavonoids that contain multiple OH substitutions have very strong antioxidant activities against peroxyl radicals. For example, the $ORAC_{ROO\cdot}$ activities of myricetin, quercetin, luteolin, fustin, eriodictyol, and taxifolin were 4.32, 3.29, 3.57, 3.91, 3.41, and 3.59 Trolox equivalents, respectively, whereas α-tocopherol, ascorbic acid, β-carotene, GSH, uric acid, and bilirubin were reported to have $ORAC_{ROO\cdot}$ values of 1.0, 0.52–1.12, 0.64, 0.68, 0.92, and 0.84 Trolox equivalents, respectively.[36,43] This observation means that the stoichiometric factor (i.e., the number of peroxyl radicals trapped per molecule of antioxidant) of these flavonoids is about 6–9, since the stoichiometric factor of Trolox is 2.[57] Therefore, one question that must be addressed is whether these flavonoids are better antioxidants than other common antioxidants, such as α-tocopherol and ascorbic acid. We would suggest that these flavonoids are as good as or better than α-tocopherol and ascorbic acid in terms of their antioxidant activity; transition metal-induced prooxidant actions of ascorbic acid[58] and α-tocopherol[44,45,59] have also been described. Using Cu^{2+}-H_2O_2 in the ORAC assay, it was also found that ascorbic acid acted as a prooxidant and the $ORAC_{OH\cdot}$ values of Trolox and uric acid also decreased with concentration after they reached their maximal $ORAC_{OH\cdot}$ values.[46]

The copper-initiated prooxidant actions of flavonoids and other antioxidants including ascorbic acid and α-tocopherol may not be important in vivo, where copper ion will be largely sequestered, except perhaps in certain metal overload diseases. The prevention of iron-increased lipid peroxidation in hepatocytes by some flavonoids including quercetin has been reported.[26,27] The protection provided by fruits and vegetables against diseases, including cancer and cardiovascular diseases, has been attributed to the various antioxidants, including flavonoids, contained in these foods. The flavonoids rutin and quercetin, when added to the diet, have been recovered in substantial concentrations in rat plasma.[25] An epidemiological study demonstrated that flavonoid intake was significantly inversely related to mortality from coronary heart disease and of borderline significance ($p < .08$ for trend) in relation to the incidence of a first fatal or nonfatal myocardial infarction.[60] The ''French Paradox,'' a fact that the French follow a lifestyle more inclusive of risk factors for coronary heart disease but have a low incidence of this disease,[61] has been attributed to the red wine consumed by the French. Red wine has a much higher antioxidant capacity than white wine because of its high flavonoid content.[35,62]

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ABBREVIATIONS

ORAC—oxygen radical absorbance capacity
AAPH—2,2′-azobis(2-amidino-propane) dihydrochloride
β-PE—β-phycoerythrin
Trolox—6-hydroxy-2,5,7,8-tetramethyl-2-carboxylic acid
$ORAC_{ROO\cdot}$—peroxyl radical absorbing activity
$ORAC_{OH\cdot}$—hydroxyl radical absorbing activity
AUC—area-under-curve
P_{max}—maximal prooxidant activity
K_m',—the concentration of a flavonoid needed to reach 1/2 P_{max}
$ABTS^{\bullet+}$—2,2′-azinobis(3-ethylbenzothiazoline)-6-sulfonate radical cation
GSH—glutathione